Chapter 4

Imaging Chambers for *Arabidopsis* Seedlings for Mitotic Studies

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Abstract

Flowering plants evolved away from creating centrosomes or conventional microtubule organizing centers. Therein, plants have posed a long-standing challenge to many of the conventional ideas for mitotic spindle construction and the process of chromosome segregation. The *Arabidopsis* seedling has emerged as a leading model for plant cell biological studies of the cytoskeleton and vesicle trafficking. Here we describe methods for creating a reusable chamber for mitotic studies in both seedling root and shoot cells with instruction for best practices with conventional microscopic techniques.

Key words *Arabidopsis* mitosis, Kevin, Seedling imaging chamber

1 Introduction

The *Arabidopsis* seedling is a leading model system for studies of cell biological phenomena in flowering dicot plants. The genetic resources and small stature of the seedling provide advantages for studying a wide variety of cellular functions in the context of the developing organism. Developmental studies examining the timing and positions of cell division in the root and apical meristems have used both ad hoc and more formally engineered methods [1] for identifying when and where cell division occurs [2, 3]. Cell biological explorations of mitosis, using high-resolution imaging techniques, have been less frequent in *Arabidopsis*, with more focus on the later events related to the establishment and formation of the cell division plane [4–6].

The fundamental mechanistic details of plant spindle formation and chromosome segregation are not well explored and scarcely appear in popular reviews of mitosis beyond the admission that flowering plants do not have centrosomes or conventional microtubule organizing centers [7–10]. Experiments performed in *Haemanthus* (tiger lily) endosperm cells [11, 12], *Tradescantia* (spiderwort) stamen hair cells [13], and *Nicotiana* (tobacco) cells

Edward H. Hinchcliffe (ed.), *Mitosis: Methods and Protocols*, Methods in Molecular Biology, vol. 2415,
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[14, 15] still constitute a large portion of the reviewed materials owing to their experimental accessibility for specific questions. Efforts in maize, a classic genetic system for plant studies, have focused more attention on meiotic processes than mitosis with a significant history developed around the control and function of centromeres [16, 17]. The development of liverwort and bryophyte (moss) systems, notably *Marchantia* and *Physcomitrella*, respectively, that feature both rapid molecular manipulation and highly predictable cell divisions has driven more direct study of the mitotic apparatus and the mechanics of chromosome segregation with a more conventional spindle architecture [10, 18–21]. Studies using *Arabidopsis* have appeared sporadically, with emphasis on signaling mechanisms surrounding the entrance to division, the positioning of microtubule nucleation complexes, and microtubule orientations in late telophase [4, 6, 8].

Several major factors impact the collection of high-resolution image data from flowering plants such as *Arabidopsis* [22, 23]. Most prominently, the plant cell wall is both highly refractive [24] and optically active in the rotation of polarized light. Most plant tissues also exhibit some level of autofluorescence across a broad spectrum of excitation and emission wavelengths. Finally, most plant cells undergoing mitosis are large enough that the nucleus or free chromosomes can move several microns within the axial focus of the microscope over a few seconds, requiring volume-based imaging methods. The combined effect is to make high-resolution (i.e., high numerical aperture) imaging a challenge from cell to cell [25]. Focusing through the cell wall, with refractive indexes varying between 1.39 and 1.45 and thickness varying over a wide range for different cell types, means that the exact focal point and quality of the diffraction-limited excitation or emission will change throughout the field. Moreover, the laser illumination from a point-scanning confocal microscope nearly always retains a high degree of polarization, a characteristic that can be reinforced or redirected as the light traverses the wall material to create a focal volume. The practical effect is that widefield fluorescence techniques and computational deconvolution rarely yield acceptable image data for analysis of live cells and confocal imaging often shows anomalous features in reconstructions of image stacks. The optical activity of the cell wall additionally hampers the application of super-resolution techniques that require polarized illumination to produce structured illumination [26].

The most successful high-resolution fluorescence methods for mitotic studies in flowering plants center on scanning confocal imaging techniques [23]. Chemically fixed specimens that have been immuno-labeled for molecules of interest and mounted in a medium that matches the expectations of current oil immersion lenses will provide the best resolution for light microscopy. For questions that can be approached with these methods, immunohistological techniques have been published that provide excellent

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guidance for root and some leaf materials [27]. For live cell imaging, both point-scanning and spinning disk confocal microscopes have been successfully deployed for determining the spatial and temporal changes occurring during mitosis [6, 28]. The choice of technique often depends upon the tissue type, molecular probes, and specific questions being posed for the study. Light-sheet microscopy will add substantively to the repertoire of methods [29] available to plant cell biologists (cites) investigating mitotic events but has yet to appear in a viable commercial platform for high-resolution plant studies.

While the *Arabidopsis* epidermal hypocotyl cells have become a leading model for plant cytoskeletal biology, mitotic division in the hypocotyl is extremely rare. Therefore, proliferating epidermal cells (Fig. 1a) in the margins of the expanding cotyledon (embryonic leaf), the petiole (connects cotyledon to seedling), and the emerging apical meristem are targets for mitotic studies. Cells in these tissues do not undergo cell division in an entirely predictable manner, so holding seedlings in the dark until 2–6 h before imaging or selecting seedlings that are coming out of the dark phase of a diurnal light cycle can improve the chances of capturing cells going into mitosis. Cells from aerial parts of the seedling are robust to seedling manipulations such as mounting or drug treatment but are often difficult to position with respect to the optical pathway of the microscope.

Alternatively, epidermal root cells between the meristem and growth zone (Fig. 1b) undergo mitotic divisions with more regularity and typically divide along a predicable cell orientation. The *Arabidopsis* root is, however, delicate and prone to damage when taken off of solid growth medium for imaging in liquid media, often necessitating the use of special imaging chambers. Moreover, functional fluorescent tubulin probes have been difficult to create for the root, leading to a reliance on fluorescent MAPs for reporting the positions and dynamic properties of root microtubules [30].

To facilitate high-resolution live cell imaging of the plant root and cotyledon cells undergoing mitosis, we developed a relatively simple chamber for seedling germination that transfers directly and repeatedly between plant incubator and microscope stage (Fig. 1). The chamber may be constructed without special appliances or materials and serves to robustly handle seedlings through 6–10 days of growth.

2 Materials

1. Liquid medium: 0.5× Murashige and Skoog (MS) medium [31] using 2.2 g MS salts (Sigma-Aldrich) and 0.5 g MES buffer to 0.9 L of distilled water. Bring the media pH to 5.7 with KOH and the final volume to 1 L with distilled water.

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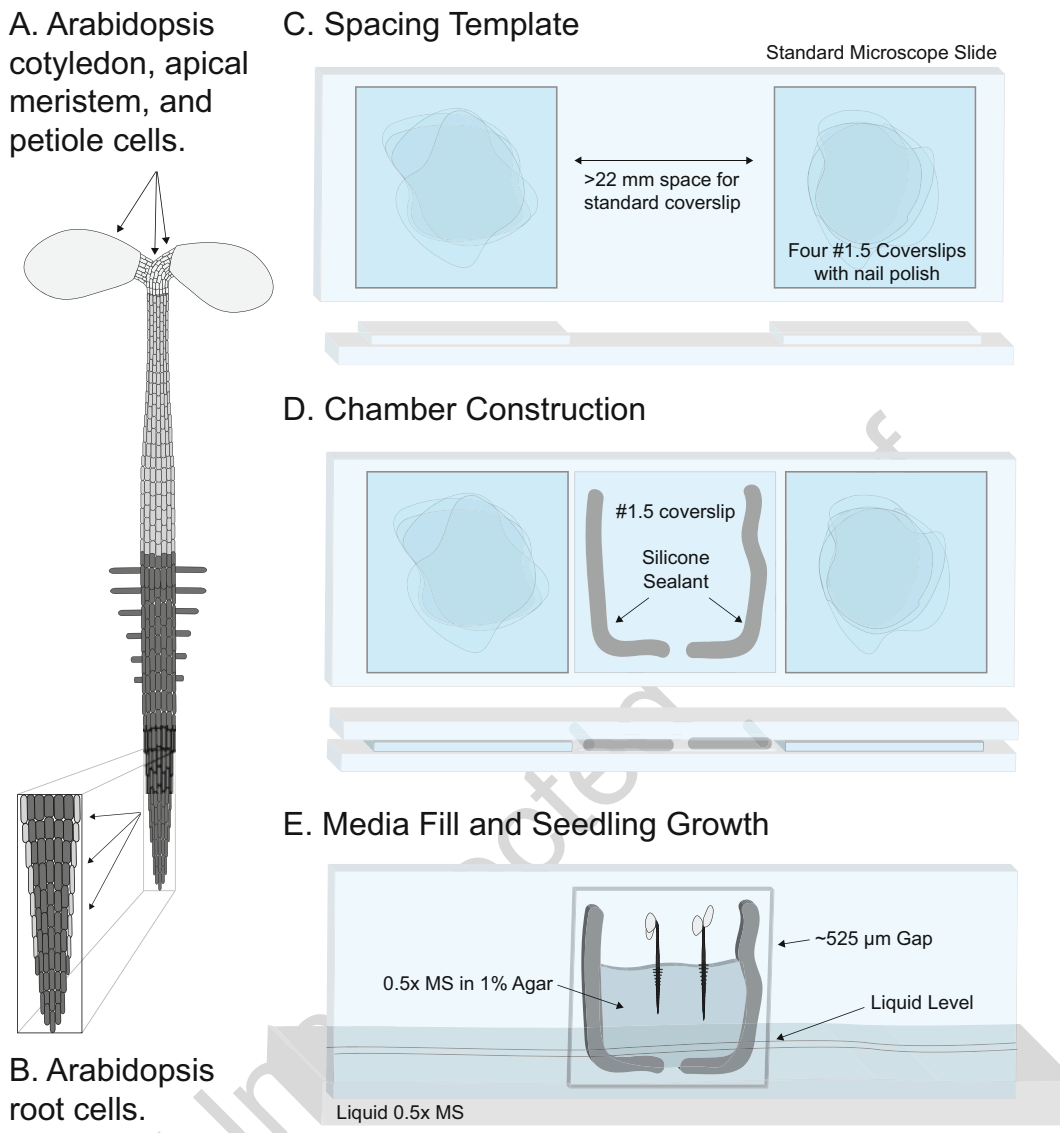

Fig. 1 Mitotic cells appear at the apical aerial portions (**a**) of the *Arabidopsis* seedling and in the beginning growth zone of the root tip (**b**). Imaging chamber is created by first making a spacing template (**c**) using stacks of four #1.5 coverslips adhered with nail polish. The chamber is constructed by placing a single #1.5 coverslip into the spacing template (**d**) and applying silicone sealant in a modified "U" pattern. A clean standard microscope slide is positioned on to the template creating a 525 µm gap for seedling germination. The chamber is filled to half-capacity with agar plant media, and sterilized seed are placed into the chamber with the agar interface (**e**). Placing chambers vertically into a liquid allows seedling growth for 6–10 days

2. Solid medium: To create 1% w/v agar medium, use 10 g plant tissue culture grade agar (Sigma-Aldrich) per 1 L media and autoclave in bottles.
3. Aliquot hot liquid media to smaller (e.g., 250 mL) autoclaved bottles that can be warmed in a microwave or heat block to re-melt media at 95–98 °C before use in chambers.

4. Seed sterilization solution: Mix hydrogen peroxide and molecular biology grade ethanol in a 30/70 (v/v) ratio. Store at 4 °C in a sealed glass bottle covered with aluminum foil to protect from light.
5. Imaging chambers are made from standard (75 × 25 mm) glass microscope slides and #1.5 weight (22 × 22 mm) coverslips obtained from any of multiple sources.
6. Glass is pre-cleaned by submerging in 100% ethanol (v/v) and air-drying immediately prior to chamber construction.
7. Chambers are constructed using a marine-grade silicone-based adhesive and should be tested for shrinkage prior to use of chambers. Gorilla 100% Silicone Sealant (The Gorilla Glue Company, Cincinnati, OH, USA) is easily applied, cures in less than 60 min, holds glass well enough to reuse chambers, and shows an acceptably low level of contraction between glass slide and coverslip.

3 Methods

3.1 Imaging Chamber Spacer Template

The simple imaging chambers are created using a template to insure the correct spacing for seedling growth between the coverglass and slide.

1. Pre-clean one standard glass microscope slide and eight standard glass #1.5 weight coverslips by immersion in 100% ethanol and air-drying.
2. The spacing template (Fig. 1) uses two stacks of four coverslips held together by optical cement or nail polish. Wearing gloves to avoid transfer of oils or dust to each coverglass, use a small drop of nail polish to attach one coverslip to one end of the glass microscope slide. Attach a second coverglass to the microscope slide with nail polish at a position greater than 22 mm away, creating a gap for the placement of a coverslip during chamber construction.
3. Repeat the process of adding coverslips to both stacks, pressing down on each side gently to create a secure contact with no air gaps. Only a small drop of adhesive is required to hold the coverslips in place, and creating a uniformly aligned stack, where no coverslip is sticking out of the stack, will ease construction of imaging chambers.
4. Allow the reusable spacing template to cure until completely rigid (i.e., 1 h). Any optical cement or nail polish on the glass slide surface between coverslip stacks should be cleaned or removed with appropriate solvent (i.e., acetone).

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3.2 Imaging Chamber Construction

The imaging chamber is constructed using a standard glass microscope slide and #1.5 (170 μm) thickness coverslip. The coverslip is affixed to the glass slide with a marine-grade silicon-based adhesive with relatively low form shrinkage.

1. Using forceps or gloves to prevent transfer of oils or dust, place a pre-cleaned #1.5 weight coverslip into the spacer template between the stacks of immobilized coverslips (Fig. 1).
2. Apply the silicone marine-grade sealant in a modified "U" shape around the periphery of the pre-cleaned coverslip, leaving a 1–2 mm gap at the base of the "U" shape (Fig. 1). The sealant should be applied in a careful bead to minimize the footprint and prevent closure of the gap in **step 3**.
3. Before the sealant has set, place a pre-cleaned standard glass microscope slide over the spacing template and in contact with the sealant (Fig. 1). Press the slide to insure that it is seated evenly on both stacks of affixed coverslips.
4. Allow the chamber to cure for 45—60 min in the spacer template before removing and use. If the sealant does not shrink, the chamber will have an internal gap of 520–550 μm, and the coverslip will remain entirely parallel to the microscope slide.

3.3 Preparing the Imaging Chamber

1. Warm the 1% agar plant media so that it is just melted using a microwave oven or heated water bath.
2. Optionally, we have sterilized chambers by submerging in ethanol and drying in a sterile hood prior to adding media.
3. Carefully hold the chamber or seat the chamber vertically on a folded piece of parafilm to prevent the liquid media from draining out of the gap in the bottom of the chamber.
4. Using a pre-heated 1 mL syringe or manual pipettor, carefully introduce 80–100 μL of the still liquid plant medium into the chamber and allow the media to solidify. The agar-based media should fill approximately 1/2 of the chamber volume and extend into the gap at the base of the chamber (Fig. 1).
5. The media in the chambers is highly susceptible to drying. We recommend using the chambers immediately. Chambers can be stored at 4 °C in a sealed container with a wet paper towel to prevent drying prior to use.

3.4 Seed Sterilization and Seedling Germination

1. We place 1–5 *Arabidopsis* seed on to a dry, creased filter paper in a tissue culture sterile hood. Up to 500 μL of sterilization solution is pipetted on to the seed and allowed to dry in the sterile hood. The sterilization reduces the impact of rapidly growing bacteria or fungi that can be carried with the harvested seed into the imaging chamber.

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2. Seed are placed into the chamber resting on the agar interface with the air. Seed are transferred using either a sterile toothpick, wetted at the end to hold the seed, or the seed are gently tapped into the chamber using the crease in the filter paper.
3. Chambers are placed in a sealed bag and placed in a small lightproof box for storage at 4 °C for 24–48 h. The cold treatment increases the synchrony of germination and early growth.
4. After cold treatment, the chamber is set upright into a recycled microscope slide box or other container where the open space at the bottom of the chamber can be placed in contact with water or media in the bottom of the container (Fig. 1e). Wicking of the liquid through the open space in the bottom of the slide into the agar media will prevent the media from drying and provide a constant level of hydration for the seedling(s). The chamber and container are kept in a transparent bag to prevent the liquid in the container from drying out or concentrating due to evaporation.
5. Imaging chambers are moved to plant incubators for germination and growth under light regimes and temperatures that are commensurate with the experimental goals. For imaging the growing root tip, the chambers are tilted ~10° from vertical so that the root tips will grow against the coverslip surface.
6. For continuous dark growth, chambers containing seed taken from the 4 °C cold treatment are placed in the incubator under lighted conditions for 1–4 h prior to being placed into a lighttight container. The light treatment, together with the change in temperature, results in more consistent germination times.
7. The chambers are kept for as long as 6 days for continuous dark-grown, and for 10 days, for light-grown and light-cycled seedlings.

3.5 Imaging Fluorescent Histone H2B Plant Lines in Root Cells

Identifying pre-mitotic cells for imaging in animal systems is greatly facilitated by changes in cell morphology and adhesion (e.g., rounding up) during late S-phase and G2. Plant cells have no correlate changes in cell shape and do not typically undergo divisions in clear temporal progressions. Several FUCCI-related markers have been used to indicate when plant cells have entered S-phase [32], though many S-phase cells in plant tissues do not progress through to mitosis, opting for endoreduplication. Therefore, we have used a histone H2B fluorescent protein fusion as a marker for finding prometaphase cells prior to nuclear envelope breakdown and spindle formation (Fig. 2).

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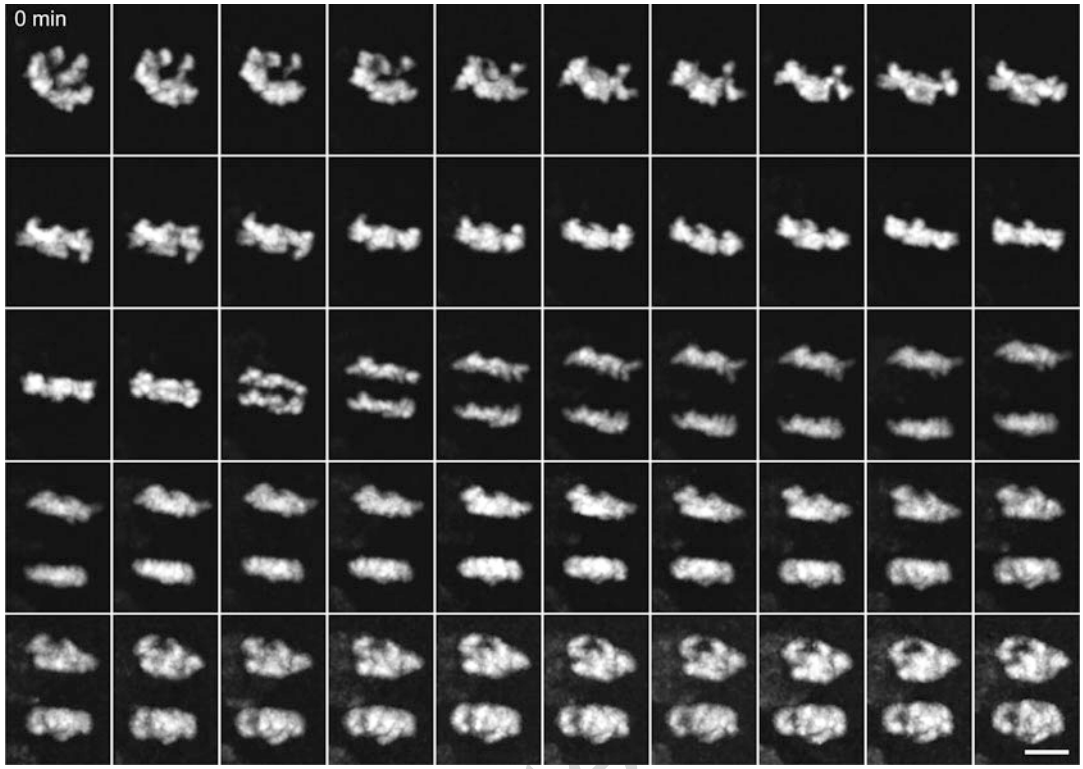

Fig. 2 Spinning disk confocal microscopy of mCherry-H2B imaged in *Arabidopsis thaliana* root cell undergoing mitosis. Time-lapsed images taken at 1.5 min intervals created from 40 frame Z-series (0.2 μm steps) arranged in rows from left to right. A 60× silicone oil immersion objective was used with 80 ms exposures on an Olympus OSR spinning disk confocal microscope using a Hamamatsu sCMOS V3.2 camera. The 50-frame series shows chromosomes just at nuclear envelope breakdown followed by congression to the metaphase plate, anaphase, and the decondensation of the chromatin during nuclear reformation. Scale bar = 5 μm

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1. Growing root tips expressing an mCherry-histone H2B fusion protein under the control of a constitutive CaMV35S promoter (courtesy of Mark Estelle) show changes in nuclear morphology prior to mitosis.
2. Take the chamber out of the incubator and gently dry the coverslip face with a lens-cleaning tissue.
3. For live cell imaging, the refractive index changes as light passes through the coverslip and into the root are best matched by water immersion, silicone oil immersion, or glycerol immersion objective lenses with effective numerical apertures from 1.1 to 1.31. Conventional oil immersion objectives, with a numerical aperture above 1.3, will show rapidly increasing aberration when focusing beyond the first 10 μm from the coverslip surface.

4. If at all possible, choosing an objective lens with a rotating correction collar will greatly improve the achievable contrast for the specimen. Adjusting the collar to find the brightest possible image at a depth that is midway through a prescribed specimen will suffice for live cell imaging purposes.
5. Mitotic progression occurs over a 10–15 time course in wild-type *Arabidopsis* root cells where the choice of image acquisition time, number of images in an axial stack, and time interval between frames will depend upon the experimental goals.
6. Note that most nuclei in epidermal cells appear at the far side of the cell and complete mitosis in a position that depends upon cell geometry. The post-mitotic formation of the cell wall tends to attach at the far side of the cell and proceed bidirectionally toward the outer face of the cell, necessitating a volume-based imaging approach for capturing key events through the process.
7. Note that most common lines expressing a fluorescent protein fused to alpha or beta tubulin do not show enough incorporation of the probe in root cells for imaging microtubules directly. Probes such as End Binding 1 (EB1b-GFP; cite) and a microtubule binding domain taken from animal cells (MBD-GFP; cites) have some use for mitotic studies in root cells.

3.6 Imaging Mitosis in Seedling Cotyledon and Apical Meristem

The appearance of mitotic figures in the accessible aerial portions of the seedling is less predictable than in the growing root tips. Keeping seedlings in the dark until 1–3 h prior to imaging will induce the cotyledons to undergo significant developmental changes related to de-etiolation. Those changes will include cell divisions in some marginal cells where the distance between coverslip and mitotic spindle can be minimized (Fig. 3).

Increased frequencies of division are also observed when seedlings are coming out of the first day-night light cycle. Tilting the chambers 10° in the direction of the microscope slide will improve the chances of having cells in the cotyledon or apical meristem close the coverslip.

1. To accommodate high-numerical aperture lenses, fill the upper portion of the chamber with sterilized tap water or plant media at least 30 min prior to imaging. This can include pharmacological treatments, live cell stains, or other experimental treatments. Neat distilled water is not recommended as it will osmotically shock the plant requiring a longer recovery time.
2. Identify cells that are as close to the coverslip surface as possible to improve the fidelity of the imaging light path. Positioning mitotic cells close to the coverslip for live cell imaging is made difficult by the shape of the organs, often necessitating longer working distance objective lenses with lower working

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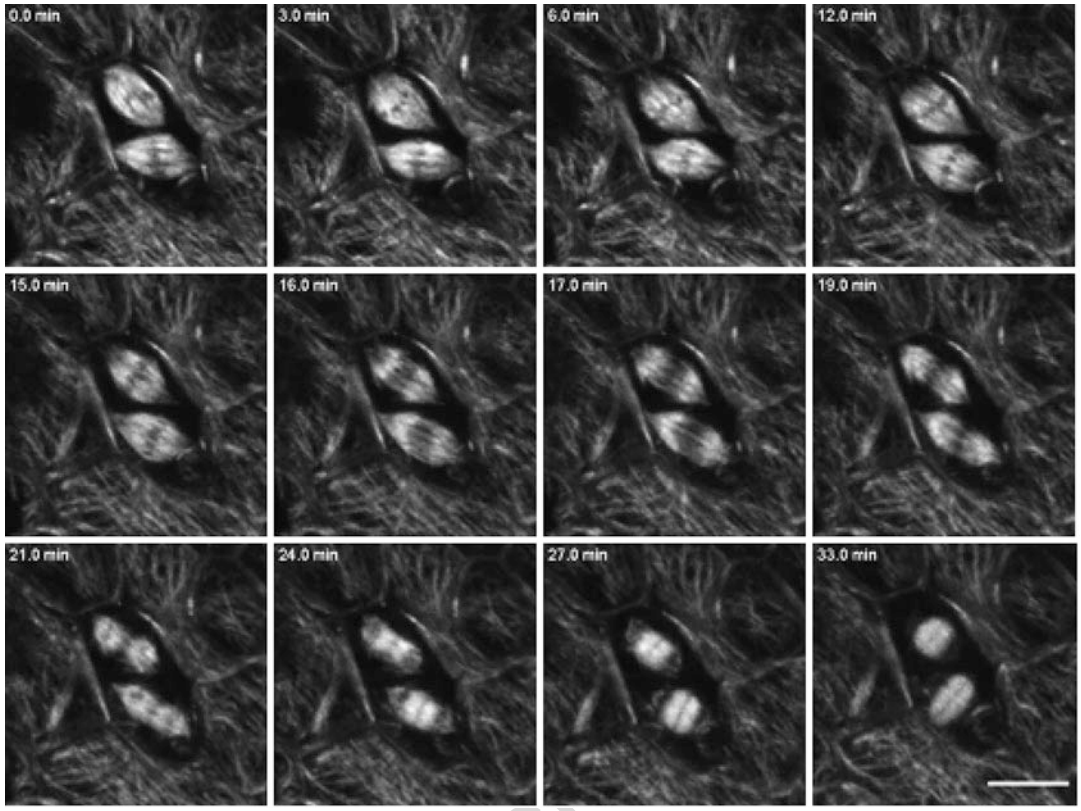

Fig. 3 Selected time points from spinning disk microscopy of Eb1b-GFP imaged in *Arabidopsis thaliana* cotyledon epidermal cells undergoing mitosis. Time-lapsed images of two adjacent cells made from 40-frame Z-series (0.2 μm steps) arranged in rows from left to right. A 60× silicone oil immersion objective was used with 80 ms exposures on an Olympus OSR spinning disk confocal microscope using a Hamamatsu sCMOS V3.2 camera. Series includes mitotic congression to the metaphase plate, anaphase, and the formation of the plant phragmoplast array associated with formation of the new cell wall. Scale bar = 5 μm

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numerical apertures. In this circumstance, conventional oil immersion objective lenses perform substantially worse than water, silicone oil, or glycerol immersion objective lenses owing to the spherical aberration. Multi-color imaging suffers dramatically for both axial and lateral colocalization.

3. Using a spinning disk or point-scanning confocal microscope will provide a starting point for gaining contrast in the cotyledon or early apical meristem. Because of the autofluorescence in these aerial tissues, we image control plants, not expressing fluorescent proteins, to determine the degree to which the autofluorescence is contributing to the final image. Highly structured autofluorescence (e.g., background shapes) or a standard deviation of the local mean values more than 10–15% of the expected signal from the fluorescent proteins should initiate a search for more restrictive band-pass filters or using different fluorescent probes.

4. Mitotic progression in the aerial parts of the seedling occurs over a 15–25 time course in wild-type *Arabidopsis* where the choice of image acquisition time, number of images in an axial stack, and time interval between frames will depend upon the experimental goals.

3.7 Alternative Imaging Methods

Techniques other than confocal microscopy can be used successfully to image mitosis in *Arabidopsis* seedlings but require a higher level of familiarity with both the instrumentation and the specific cell types. Conventional widefield fluorescence imaging of living specimens rarely produces useful data because the out-of-focus fluorescence from the mitotic spindle typically overwhelms the in-focus image. Computational image deconvolution of widefield fluorescence images also fails under most circumstances because of the inhomogeneous optical aberrations introduced by the cell wall material. Multi-photon imaging in plant systems offers the advantage of imaging deeper and with potentially less overall photodamage to the cells when compared to confocal imaging [33]. However, the loss of lateral resolution, owing to the longer wavelength excitation, and the difficulty in controlling the excitation energy through depth make this technique challenging. Super-resolution STED techniques have shown very limited success in plant systems, with newer time-gated systems providing information for cortical structures [34]. Light-sheet microscopy offers a much gentler means of extracting fluorescent signals and should be markedly faster than point-scanning confocal microscopy [29]. The convex shape of the plant and the optical properties of the plant cell wall make current light-sheet microscopes difficult to apply for mitotic studies where shading and distortions often preclude collection of data. Newer light-sheet imaging designs with numerical apertures closer to 1.33 will likely improve the use of this technology dramatically. Total internal reflectance (TIRF) is not an appropriate choice for mitotic studies because the depth of field and position of the mitotic spindle with reference to the coverslip surface extend well beyond the evanescent field generated under nearly all circumstances. Related techniques have been very successful for imaging cortical structures when cells are appressed to the coverslip [35].

For bright-field imaging of mitosis in plant cells, phase-contrast microscopy does not have the same utility as it does for animal cells because the cell wall accentuates the phase differences in the light path, increasing the flare or optical ringing in the image. Differential interference contrast (DIC) imaging works surprisingly well for visualizing the segregation of chromosomes where a monochromatic illumination filter is strongly encouraged (e.g., the green filter in some widefield light paths) to limit dispersion that will reduce contrast in the image.

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4 Notes

1. The largest microscope-independent factor impacting data quality is the position of the cell relative to the objective lens front element. The closer the mitotic cell is to the lens, the better the signal collection. Moreover, keeping the outer (periclinal) cell wall perpendicular to the optical axis, rather than at an angle, reduces the negative optical effects introduced by the cell wall materials.
2. Because these plant tissues do not undergo a synchronous or well-predicted temporal pattern of cell divisions, using a light regime to improve the probability of finding cells going into mitosis markedly increases the likelihood of success.
3. Water, glycerol, and silicone oil immersion objective lenses provide consistently better three-dimensional image volumes than conventional oil immersion objective lenses. For specimens in these chambers, a numerical aperture above 1.35 will produce more aberrations, degrading the final image, than lenses designed to work with water-based media. Water immersion objectives are significantly more sensitive to coverslip flatness in the optical path requiring careful mounting of the coverslip with spacers when using this chamber system.

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